JOURNAL OF CLINICAL MICROBIOLOGY, May 1997, p. 1122–1130 Vol. 35, No. 5
0095-1137/97/$04.00+0

Serological Diagnosis of Hantavirus Infections by an Enzyme-Linked Immunosorbent Assay Based on Detection of Immunoglobulin G and M Responses to Recombinant Nucleocapsid Proteins of Five Viral Serotypes

FREDRIK ELGH,[1]* ÅKE LUNDKVIST,[2] OLEG A. ALEXEYEV,[1,3] HANS STENLUND,[4] TATJANA AVSIC-ZUPANC,[5] BRIAN HJELLE,[6] HO WANG LEE,[7] KENNETH J. SMITH,[8] RAIJA VAINIONPÄÄ,[9] DONNA WIGER,[10] GÖRAN WADELL,[1] AND PER JUTO[1]

Departments of Virology[1] and Statistics,[4] University of Umeå, Umeå, and Swedish Institute for Infectious Disease Control and Department of Virology, Karolinska Institute, Stockholm,[2] Sweden; Department of Infectious Diseases, Samara State Medical University, Samara, Russia[3]; Institute of Microbiology, Medical Faculty, Ljubljana, Slovenia[5]; Department of Pathology, University of New Mexico, Albuquerque, New Mexico[6]; Asan Institute for Life Sciences, Seoul, South Korea[7]; Department of Medicine, Emory University School of Medicine, Atlanta, Georgia[8]; Department of Virology, University of Turku, Turku, Finland[9]; and Department of Virology, National Institute of Public Health, Oslo, Norway[10]

Received 14 June 1996/Returned for modification 17 September 1996/Accepted 27 January 1997

Worldwide, hantaviruses cause more than 100,000 human infections annually. Rapid and accurate methods are important both in monitoring acute infections and for epidemiological studies. We and others have shown that the amino termini of hantavirus nucleocapsid proteins (Ns) are sensitive tools for the detection of specific antibodies in hantavirus disease. Accordingly, we expressed truncated Ns (amino acids 1 to 117) in *Escherichia coli* from the five hantaviruses known to be pathogenic to man; Hantaan (HTN), Seoul (SEO), Dobrava (DOB), Sin Nombre (SN), and Puumala (PUU) viruses. In order to obtain pure antigens for use in an enzyme-linked immunosorbent assay (ELISA), the recombinant proteins were purified by polyhistidine-metal chelate affinity chromatography. Polyclonal animal antisera and a panel of serum specimens from hantavirus-infected individuals from Scandinavia, Slovenia, Russia, Korea, China, and the United States were used to evaluate the usefulness of the method. With both human and animal sera, it was possible to designate the antibody response into two groups: those with HTN, SEO, and DOB virus reactivity on the one hand and those with SN and PUU virus reactivity on the other. In sera from Scandinavia, European Russia, and the United States, the antibody response was directed mainly to the PUU and SN virus group. The sera from Asia reacted almost exclusively with the HTN, SEO, and DOB types of viruses. This was true for both the immunoglobulin M (IgM) and IgG antibody responses, indicating that this type of discrimination can be done during the acute phase of hantavirus infections. Both the HTN, SEO, and DOB virus and the PUU and SN virus types of antibody response patterns were found in patients from the Balkan region (Slovenia).

Hantaviruses, which belong to the family *Bunyaviridae*, cause two distinct, severe human infections; hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (21, 32). HFRS is characterized by fever, renal dysfunction, and hemostatic imbalance (9, 39). More than 100,000 cases are reported annually (30). Four HFRS-associated hantaviruses have been described. Hantaan (HTN) virus causes severe HFRS in Asia (23). Seoul (SEO) virus, reported to cause moderately severe HFRS, is linked to urban rats and therefore is potentially found all over the world (22). Dobrava (DOB) virus, which was isolated on the Balkan Peninsula, has been found to be an important cause of the more severe type of HFRS in this region (4, 4a, 31a). Puumala (PUU) virus, the cause of a milder form of the disease (nephropathia epidemica [NE]), is found in Scandinavia, western Russia, the Balkan region, and several central European countries (5, 24). The mortality rate from infections caused by HTN virus is reported to be 3 to 10%. For SEO viruses this figure is substantially lower (~1%), whereas for PUU virus infection it is approximately 0.2% (30, 39). HPS, a recently discovered disease entity in the Americas, involves adult respiratory distress syndrome with a high mortality rate (~50%) and is caused by Sin Nombre (SN) virus and related agents (10, 32).

The hantaviruses are 90 to 120 nm in size and enveloped and have a tripartite, negative-stranded RNA genome which encodes an RNA-dependent RNA polymerase, two envelope glycoproteins (G1 and G2), and a nucleocapsid protein (N) (30). Rodents are the natural reservoirs of hantaviruses, and transmission to humans is believed to occur mainly via aerosolized animal excreta (30). The principal rodent hosts for HTN, SEO, PUU, DOB, and SN viruses are *Apodemus agrarius*, *Rattus norvegicus*, *Clethrionomys glareolus*, *Apodemus flavicollis*, and *Peromyscus maniculatus*, respectively (4–6, 17, 23).

The clinical diagnosis of hantavirus infections has routinely been confirmed by immunofluorescence antibody assay (IFA) or enzyme-linked immunosorbent assay (ELISA) with native viral antigens (24, 34, 35). Due to the hazardous nature of hantaviruses, their slow replication, and low and variable yield in cell culture, recombinant hantavirus proteins have been produced for use as antigens in serological assays (13, 14, 18, 19, 43–45). Hantavirus N elicits a strong humoral immune response in infected patients and immunized animals (11–13,

* Corresponding author. Mailing address: Department of Virology, Umeå University, S-901 85 Umeå, Sweden. Phone: 46-90-785 13 06. Fax: 46-90-12 99 05. E-mail: Fredrik.Elgh@climi.umu.se.

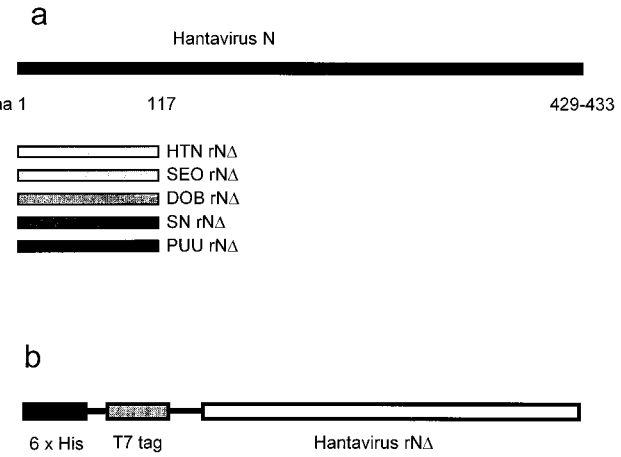

FIG. 1. (a) Schematic representation of the 433-aa hantavirus N compared to the 117-aa rNΔ proteins. (b) Schematic representation of the *E. coli*-produced hantavirus rNΔ with the amino-terminal histidine tag (6× His) and the T7 tag MAb epitope (T7 tag).

27, 31, 40). High levels of immunoglobulin M (IgM) antibody to the PUU virus N have been detected at the onset of NE, which indicates that it is suitable as the sole antigen for serodiagnosis (11, 13, 27). We and others have shown that the major humoral response to hantavirus N resides within the amino-terminal portion of the protein (12, 18, 28, 31).

In this work we present the efficacy of an ELISA for the serological diagnosis of hantavirus infections occurring on the Eurasian and American continents. The ELISA is based on *Escherichia coli* expressed amino-terminal recombinant Ns (rNs) from five different viral serotypes pathogenic for humans.

MATERIALS AND METHODS

Cloning of amino-terminal hantavirus open reading frames and construction of expression vectors. Primers, designed according to previously published gene sequences, were used to amplify hantavirus HTN virus strain 76/118-, SEO virus strain SR-11-, DOB virus strain 3970-, SN virus strain CC107-, and PUU virus strain Sotkamo-specific cDNAs. These primers covered the nucleotide sequences encoding amino acids (aa's) 1 to 117 of their Ns (hantavirus-specific primer pairs were as follows: for HTN virus, ATGGCAACTATGGAGGAATTACAGA and TGCTGTCTGTCCTGTAGGTTCATCAAT; for SEO virus, ATGGCAACTA TGGAAGAAATCCAGA and AGCTGTCTGTCCTGTAGGTTCATCAAT; for DOB virus, ATGGCAACATTAGAGGAACTCCAAA and TGCAGTTTG CCCTGTAGGTTCATC; for SN virus, ATGAGCACCCTCAAAGAAGTGC AAG and AGCAGTCTGACCACTCGGCTCTTC; and for PUU virus, ATGA GTGACTTGACAGATATCCAAG and TGC TGTTTGGCCACTTGGTTCT TC) (1, 3, 25, 38, 42) (Fig. 1a). The primer design included the addition of endonuclease restriction sites in the ends of amplimers to facilitate cloning into the pRSETA polyhistidine fusion protein *E. coli* expression vector (R&D Systems Europe Ltd., Oxford, United Kingdom). The fusion proteins expressed by this vector contain six amino-terminal histidine residues for convenient affinity purification and an 11-aa epitope (encoded by the bacteriophage T7 gene 10) for specific monoclonal antibody (T7 tag MAb; Novagen Inc., Madison, Wis.) detection of the expressed protein (Fig. 1b).

Genetic characterization of expression vector-insert junctions. Expression vectors and inserts were analyzed with appropriate restriction endonucleases (Boehringer Mannheim GmbH, Mannheim, Germany) and by DNA sequencing (Pharmacia ALF and T7 sequencing kit; Pharmacia Biotech Norden AB, Sollentuna, Sweden).

Expression and purification of truncated rNs. Expression of the plasmid constructs transformed into *E. coli* BL21(DE3) (Novagen) was induced by the addition of 1.0 mM isopropyl β-D-thiogalactopyranoside at an optical density at 600 nm of 0.7. Cultures were then allowed to grow for 3 h at 37°C. The proteins were harvested by mild lysis of the induced cells (250 μg of lysozyme per ml, 1.25 mg of deoxycholate per ml, 10 μg of RNase A per ml, 5 μg of DNase I per ml), the lysed cells were centrifuged at 10,000 × *g*, and thereafter, supernatants containing recombinant protein were recovered. Expressed polyhistidine-containing fusion proteins were then affinity purified over a chromatography column containing Ni^{2+} (QIAGEN, GmbH, Hilden, Germany). The recombinant proteins were purified under denaturing conditions by using a buffer containing 0.1 M NaH_2PO_4 and 8 M urea with decreasing pH in three steps. Eluates were analyzed by separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with 15% polyacrylamide gels (Fig. 2). Affinity-purified truncated rN (rNΔ) fusion proteins were stored at −20°C in the final elution buffer. Protein concentrations of the antigen preparations were measured by a Coomassie brilliant Blue G-250-based colorimetric assay (Bio-Rad Laboratories, Hercules, Calif.).

Animal sera. Antisera to HTN virus (strain 76/118 [23]), SEO virus (strain 80/39 [22]), and PUU virus (strain K-27 [40]) were produced by a single intramuscular inoculation of New Zealand White rabbits with cell culture-propagated viruses (37). Antiserum to DOB virus (strain 3970 [4]) was produced by intranasal inoculation of New Zealand White rabbits as described previously for chinchilla rabbits (33), and serum was collected 2 months after infection. A rabbit polyclonal rN antiserum was raised by intramuscular immunization of 200 μg of an SDS-polyacrylamide gel-purified PUU virus Sotkamo rN in Freund's complete adjuvant. Two booster injections with the same antigen dose were given at 1 and 3 months postimmunization. Polyclonal mouse anti-SN virus 3H226 rN serum was raised by immunizing animals three times with full-length rN (maltose-binding fusion protein expressed in *E. coli*) and mouse polyclonal anti-PUU virus Sotkamo rN serum by immunizing animals three times with full-length rN (10a, 16).

Sera from subjects with HFRS and HPS. Sera from immune individuals or patients with acute HFRS or HPS infection were obtained from Sweden (n = 206), Norway (n = 21), Finland (n = 18), western Russia (Samara region) (n = 49), Slovenia (n = 46), Korea (n = 10), China (n = 17), and the United States (New Mexico and Arizona) (n = 13).

Control sera. Sera that did not reveal IgG reactivity in the PUU virus IFA were collected from 52 healthy blood donors and 48 patients suffering from acute viral infections other than HFRS or HPS (i.e., Epstein-Barr virus, cytomegalovirus, and adenovirus infections).

rNΔ ELISA. Microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 0.2 μg of hantavirus antigen per well, and the plates were incubated overnight at room temperature. Each of the five recombinant hantavirus antigens was diluted in carbonate buffer (pH 9.6). The five antigens were coated in separate columns so that all five antigens and controls containing only coating buffer were present on the same ELISA plate. The fusion proteins expressed by pRSETA contain an 11-aa T7 tag MAb epitope (Novagen) (Fig. 1). This epitope was used to determine the relative number of epitopes present in the five hantavirus rNΔ antigen preparations. By using chessboard titrations of the rNΔ antigens and the T7 tag MAb, it was then possible to achieve similar amounts of the different hantavirus rNΔ antigens in the ELISA wells of the ELISA plates. The plates were washed in deionized water and were then blocked with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 4% defatted milk powder for 30 min at room temperature. After the plates were washed in deionized water, 100 μl of serum (diluted 1/400 in PBS with 0.1% Tween 20, 2% defatted milk powder, and *E. coli* antigen extract [20 μg/ml]) was added and the plates were incubated at 37°C for 1 h. Each serum sample was tested in duplicate wells containing all five hantavirus antigens and control wells containing no antigen. The control wells were included as a monitor for nonspecific activity. After four washes in deionized water, either horseradish peroxidase (HRP)-conjugated goat anti-human IgG (catalog no. A-6029; Sigma) at a dilution of 1/10,000 or HRP-conjugated goat $F(ab')_2$ fragment anti-human IgM (catalog no. A-4290; Sigma) at a dilution of 1/5,000 in serum dilution buffer was added to each well, and the plate was incubated at 37°C for 1 h. After four washes in deionized water and 15 min of incubation with 100 μl of substrate, tetramethylbenzidine (TMBLUE; ANL-produkter, Stockholm, Sweden), the reaction was stopped with 50 μl of 1 M H_2SO_4. Antibody activity was expressed in arbitrary units (AU) as the net absorbance at 450 nm (i.e., absorbance of antigen-sensitized well − absorbance of control well) divided by the mean net absorbance of two positive control wells and then multiplied by a factor of 100. Serum samples were pretreated with rheumatoid factor (RF)-absorbent (Behringwerke AG, Marburg, Germany) for the hantavirus-specific IgM assays.

The T7 tag MAb was used at a dilution of 1/10,000. Rabbit and mouse antisera were endpoint titrated by ELISA by using twofold serial dilutions, and the bound antibodies were detected by HRP-conjugated anti-rabbit or anti-mouse antibodies (catalog no. P 399 and P 447, respectively; DAKO A/S, Glostrup, Denmark) at a dilution of 1/2,000. A net absorbance value of >0.15 was considered positive.

IFA. IFA was carried out as described earlier (13). Virus-infected Vero E6 cells were dried and acetone fixed onto spot slides as the antigen source. Human sera were titrated fourfold from 1/40 to 1/640, applied to the antigen, and then incubated for 30 min at 37°C. The preparations were then incubated at 37°C for 1 h with fluorescein-labeled rabbit anti-human IgG (catalog no. F 202; DAKO) diluted 1/40 in Evans blue. The presence of typical fluorescent hantavirus inclusions was the criterion for a positive result by IFA.

WB. One microgram of the purified rNΔ fusion protein preparations was separated on an SDS–15% polyacrylamide gel in a mini-PROTEAN II electrophoresis cell (Bio-Rad), and the gel was transferred by means of electroblotting to Immobilon-P membranes (Millipore Corporation, Bedford, Mass.). The filters were then incubated with patient serum (diluted 1/100 in PBS with 0.1% Tween 20, 5% defatted milk powder, and 20 μg of *E. coli* antigen extract per ml) by

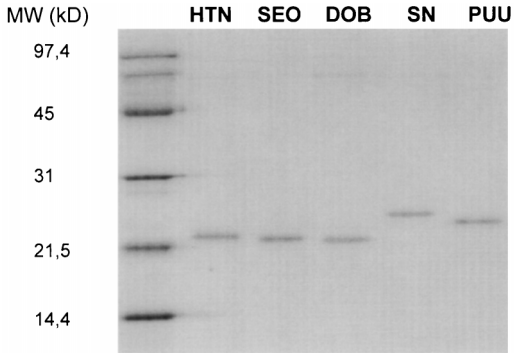

FIG. 2. Polyacrylamide gel electrophoresis of affinity-purified HTN, SEO, DOB, SN, and PUU virus truncated amino-terminal recombinant polyhistidine-containing fusion proteins produced in *E. coli*. The numbers correspond to the molecular weight markers (MW).

using a mini-PROTEAN II multiscreen apparatus (Bio-Rad). Western blotting (immunoblotting) (WB) was done according to the manufacturer's instructions (Amersham International plc, Buckinghamshire, England). HRP-conjugated goat anti-human IgG (catalog no. A-6029; Sigma) at a dilution of 1/2,000 was used to detect bound antibodies.

Amino acid sequence comparisons. Comparison of the amino acid sequences in the expressed hantavirus proteins were done by using the Clustal program in the Lasergene sequence analysis software package (DNASTAR Inc., Madison, Wis.) (15).

Statistical analysis. To compare the results of IFA, ELISA, and WB, Kendall's concordance coefficient test was used. The Spearman rank correlation test was used to show the level of correlation of reactivity toward pairs of rNΔs of different hantavirus serotypes. A multivariant analysis of variance test was used to compare the mean levels of reactivity to the five recombinant hantavirus antigens in patient sera. A linear discriminant analysis was used to illustrate the classification power of the sera. The latter statistical methods normally require distributed data, and since it is obvious from the data in Fig. 4 and 5 that the distributions are highly skewed, a log transformation of the data was necessary.

RESULTS

Cloning, expression, and purification of hantavirus amino-terminal Ns encoding open reading frames. Nucleotide sequence and control restriction endonuclease digests of the five hantavirus rNΔ expression vector constructs identified the expected sequences and digestion patterns. Gel electrophoresis on SDS–15% polyacrylamide gels revealed highly purified recombinant proteins of the expected sizes for all five serotypes expressed (Fig. 2). The yield of recombinant proteins per 500 ml of induced *E. coli* cells ranged from 0.7 to 7 mg for the different hantavirus rNΔs.

Amino acid sequence comparisons of hantavirus N proteins. The amino acid sequences of the five amino-terminal rNΔs showed a high degree of homology (63.2 to 82.1%) when the HTN, SEO, and DOB virus sequences were compared (Table 1). There was also considerable similarity between the PUU and SN virus Ns at the amino acid level in this region (70.9%). The amino acid sequence homology between HTN, SEO, and DOB virus Ns and SN and PUU virus Ns was lower (35.9 to 54.7%).

Rabbit and mouse antihantavirus hyperimmune sera. The polyclonal antisera produced against the cell culture-grown virus and the recombinant viral proteins were tested for their activities to the five hantavirus rNΔs. Usually, the sera showed the highest antibody titers to the homologous virus (Table 2). Higher antibody titers were obtained when animals were immunized with recombinant viral proteins than when they were immunized with cell culture-grown virus (Table 2). The mean antibody titer of the sera from animals immunized with rN was four times higher than the sera from the infected animals. The sera could be grouped into two types of antibody responses, HTN, SEO, and DOB viruses and SN and PUU viruses, when the antibody titers to the homologous antigen were compared (Table 2). With some of the sera, a substantial cross-reactivity was seen between the two groups of viruses, especially with the rabbit antiserum to PUU virus rN. With these sera the difference in endpoint titer between the different virus strains was only fourfold. The mouse sera raised against SN and PUU virus rNs discriminated best between the hantavirus rNΔ antigens (Table 2).

TABLE 1. Compilation of amino acid sequence similarity and divergence of the five rNΔs

Amino acid region and rNΔ	% amino acid sequence similarity			
	SEO virus rNΔ	DOB virus rNΔ	SN virus rNΔ	PUU virus rNΔ
aa's 1 to 117				
HTN virus rNΔ	82.1	65.8	54.7	50.4
SEO virus rNΔ		63.2	52.1	49.6
DOB virus rNΔ			41.0	35.9
SN virus rNΔ				70.9
aa's 17 to 59				
HTN virus rNΔ	83.7	81.4	44.2	44.2
SEO virus rNΔ		63.2	46.5	46.5
DOB virus rNΔ			46.5	46.5
SN virus rNΔ				74.4

HFRS and HPS patient IgG responses to truncated amino-terminal hantavirus N fusion proteins by IFA, ELISA, and WB. Sera from patients with acute hantavirus infection from Sweden ($n = 4$), the United States ($n = 2$), and Korea ($n = 2$) plus sera from four healthy Swedish blood donors were compared for their reactivities with five hantavirus antigens (HTN, SEO, DOB, SN, and PUU viruses) by IFA, ELISA, and WB (Table 3 and Fig. 3). By statistical analysis, the results of the three assays were in agreement for all five antigens (Kendall's concordance coefficient, 0.66 to 0.89; $P < 0.001$). For serum samples 4 (Sweden) and 5 (United States), it was evident that the cross-reactivity between the five hantavirus serotypes was high in all three assays used.

Hantavirus IgG and IgM antibody responses of HFRS and HPS patient sera from different regions of the world. Sera from patients in Scandinavia and Russia reacted strongest in the PUU virus ELISA, and these sera also displayed a rather high IgG antibody activity in the SN virus ELISA (Fig. 4a and b). However, the majority of these sera showed little if any activity in HTN, SEO, and DOB virus ELISAs. Sera from U.S. HPS patients gave the strongest reactions with the SN virus antigen. A substantial cross-reactivity to the PUU virus antigen was observed with these sera (Fig. 4d), and some of them reacted equally well with both antigens (data not shown). Sera of Asian origin reacted nearly equally with the HTN, SEO, and DOB virus antigens, and all except one serum sample showed almost no reactivity with either SN or PUU virus rNΔ antigens (Fig. 4e). The sera obtained from Slovenia (Balkan region) could be divided into two groups; one mimicked what was found for Scandinavian and western Russian sera and the other reacted like the Asian sera (Fig. 4c and f, respectively). This division was based on at least twofold higher reactivity to antigens from one group or the other in individual sera.

Statistical analyses confirmed these observations. By analyzing the results for sera by the Spearman rank signed test, highly positive correlations in reactivities within the HTN, SEO, and DOB virus serotype group ($r = 0.79$ to 0.94; $P < 0.0001$) and in the SN and PUU virus serotype group ($r = 0.72$ to 0.78; $P <$

TABLE 2. Reactivities of polyclonal rabbit and mouse sera to *E. coli*-produced rNΔ (aa's 1 to 117)

Antigen	Species	Reciprocal endpoint titer for the following recombinant antigen[a]:					Relative reciprocal endpoint titers for the following recombinant antigen[b]:				
		HTN virus[c] *rN*Δ	SEO virus[d] *rN*Δ	DOB virus[f] *rN*Δ	SN virus[g] *rN*Δ	PUU virus[h] *rN*Δ	HTN virus[c] *rN*Δ	SEO virus[d] *rN*Δ	DOB virus[f] *rN*Δ	SN virus[g] *rN*Δ	PUU virus[h] *rN*Δ
HTN virus[c]	Rabbit	20,480	20,480	20,480	1,280	1,280	100	100	100	6.25	6.25
SEO virus[e]	Rabbit	40,960	40,960	40,960	5,120	5,120	100	100	100	12.5	12.5
DOB virus[f]	Rabbit	10,240	10,240	20,480	640	1,280	50	50	100	3	6
SN[g] rN	Mouse	2,560	5,120	2,560	163,840	40,960	2	3	2	100	25
PUU virus[i]	Rabbit	1,280	1,280	1,280	5,120	20,480	6	6	6	25	100
PUU[h] rN	Rabbit	20,480	20,480	20,480	81,920	81,920	25	25	25	100	100
PUU[h] rN	Mouse	640	1,280	1,280	20,480	81,920	1	2	2	25	100
Not immunized	Rabbit	<40	<40	<40	<40	<40					
Not immunized	Mouse	<40	<40	<40	<40	<40					

[a] Titers of homologous virus and recombinant antigen are underlined.
[b] Titers of homologous virus and recombinant antigen are underlined.
[c] Strain 76/118.
[d] Strain SR-11.
[e] Strain 80-39.
[f] Strain 3970.
[g] Strain CC107.
[h] Strain Sotkamo.
[i] Strain K27.

0.0001) were found. No correlations of seroreactivity to antigens between the two groups were observed ($r = -0.07$ to 0.05; $P > 0.1$).

Multivariate analysis of variance revealed that the five means for the log-transformed serum IgG reactivities in the SN and PUU virus group differed significantly from the mean values for the HTN, SEO, and DOB virus group (Hotellings $T^2 = 2.64$; $P < 0.001$). The result holds true for the mean values for individual serum samples as well as for the vector for the five means.

Linear discriminant analyses of all sera except those from Slovenia revealed a significant discriminating function (Wilks lambda = 0.27; $P < 0.001$), with the help of which it was again possible to classify the sera into two groups (i.e., the HTN, SEO, and DOB virus group and the SN and PUU virus group) according to their log-transformed values of reactivity. The overall success of the classification was 99.4% correctly classified. All except one serum sample originating in Asia and one serum sample originating in Europe were correctly classified.

If the sera from Slovenia were included, it was not possible to arrive at this type of result. The reason for this is probably the presence of hantavirus serotypes from both groups in that part of the world. A classification of the Slovenian material such that sera with more than twofold higher reactivity toward the SN and PUU virus antigens than to the HTN, SEO, and DOB virus antigens were classified as belonging to the European and American sera and the remaining sera, all yielding a reactivity that was twofold higher to the HTN, SEO, and DOB virus antigens than to the SN and PUU virus antigens, were classified as belonging to the Asian sera, resulted in a 99.5% correct classification by the linear discriminant analysis.

Linear discriminant analysis of IgM reactivities of all serum samples resulted in 99.7% correct classification.

In conclusion, these findings indicate that by using amino-terminal rNΔ antigens in ELISA it is possible to group sera from patients with an acute or retrospectively diagnosed hantavirus infection into two groups: those with HTN, SEO, and DOB virus specificity and those with SN and PUU virus specificity. All sera that had IgG reactivity to the HTN, SEO, and DOB virus group had significant reactivity to all three antigens. A majority of the sera belonging to the SN and PUU virus group also revealed IgG antibody responses to both SN and PUU virus rNΔ antigens. With a few exceptions, hantavirus infections caused by viruses belonging to the HTN, SEO, and DOB virus group or the SN and PUU virus group could be distinguished by using the IgM-specific five rNΔ hantavirus protein-based ELISA for sera obtained from patients in the acute and early convalescent phase of disease (Fig. 5a to f). Control sera from the 52 healthy Swedish blood donors and the 48 patients with acute viral diseases other than infections caused by hantaviruses showed little if any activity in either the IgG or IgM ELISA based on the rNΔ hantavirus antigens.

DISCUSSION

It has been suggested that the extent of disease caused by hantaviruses in different regions of the world is only partially recognized. Among the factors that might contribute to this are the viral antigens used in the serological methods which have been used to diagnose hantavirus infections. Such antigens are produced by replication of live virus in cell culture, which is potentially hazardous and produces interbatch variations in antigenicity.

One of the aims of this study has been to develop assays that will facilitate serological investigations. Earlier, we have shown that *E. coli*-produced PUU virus N works well for the detection of virus-specific antibodies in patients with NE (11, 13). Furthermore, we found that this protein contains an immunodominant antigenic domain in its amino-terminal region (aa's 7 to 94) and that antibody assays based on this region of rN were equal in sensitivity to assays based on the full-length rN for the diagnosis of NE (12). It has also been shown that the antibody responses to the SN virus rN are mainly directed to epitopes in the amino terminus of this protein (18). We therefore decided to subclone the N amino terminus (aa's 1 to 117) encoding DNA from the five major pathogenic hantaviruses known to date, HTN, SEO, DOB, SN, and PUU viruses, into polyhistidine fusion protein expression vectors. The gene products were then purified, and these proteins were used in ELISAs. In these assays, the individual hantavirus antigens were coated at nearly equimolar concentrations by using the 11-aa bacteriophage T7 gene 10 peptide present in all fusion proteins.

TABLE 3. Comparison of IgG reactivities by IFA, ELISA, and WB[a]

Antigen	Assay method[a]	Result for the following serum sample no. for patients from the indicated region with the indicated disease:											
		1, Sweden NE	2, Sweden, NE	3, Sweden, NE	4, Sweden, NE	5, United States, HPS	6, United States, HPS	7, Korea, KHF[b]	8, Korea, KHF	9, Sweden[c]	10, Sweden[c]	11, Sweden[c]	12, Sweden[c]
HTN virus[d]	IFA	<40	40	160	40	40	<40	≥640	≥640	<40	<40	<40	<40
HTN virus rNΔ	ELISA	0	0.14	0.72	0.47	0.5	0	1.44	1.55	0.01	0.01	0.01	0.01
HTN virus rNΔ	WB	−	±	−	+	+	−	+	+	−	−	−	−
SEO virus[e]	IFA	40	160	160	160	160	<40	≥640	≥640	<40	<40	<40	<40
SEO virus[f] rNΔ	ELISA	0.09	0.19	0.72	0.59	0.44	0	1.45	1.31	0	0	0.01	0.02
SEO virus[f] rNΔ	WB	−	±	±	+	+	−	+	+	−	−	−	−
DOB[g] virus	IFA	160	160	640	160	160	40	≥640	≥640	<40	<40	<40	<40
DOB virus rNΔ	ELISA	0.09	0.15	0.79	0.3	0.45	0	1.56	1.21	0	0	0	0
DOB virus rNΔ	WB	−	±	±	±	+	−	+	+	−	−	−	−
SN virus[h]	IFA	160	160	≥640	160	160	≥640	<40	40	<40	<40	<40	<40
SN virus rNΔ	ELISA	0.24	0.65	1.35	0.67	1.43	1.76	0.02	0.00	0	0	0	0.01
SN virus rNΔ	WB	+	+	+	+	+	+	−	−	−	−	−	−
PUU virus[i]	IFA	≥640	≥640	≥640	≥640	≥640	≥640	40	40	<40	<40	<40	<40
PUU virus rNΔ	ELISA	1.41	2.13	1.44	1.64	1.48	1.81	0.08	0.05	0	0.01	0	0
PUU virus rNΔ	WB	±	+	+	+	+	+	−	−	−	−	−	−

[a] Data are presented as the reciprocal endpoint titers by IFA, as net optical density values by ELISA, and as a positive (+), weakly positive (±), or negative (−) result by WB.
[b] KHF, Korean hemorrhagic fever.
[c] Healthy control.
[d] Strain 76/118.
[e] Strain 80/39.
[f] Strain SR-11.
[g] Strain 3970.
[h] Strain CC107.
[i] Strain Sotkamo.

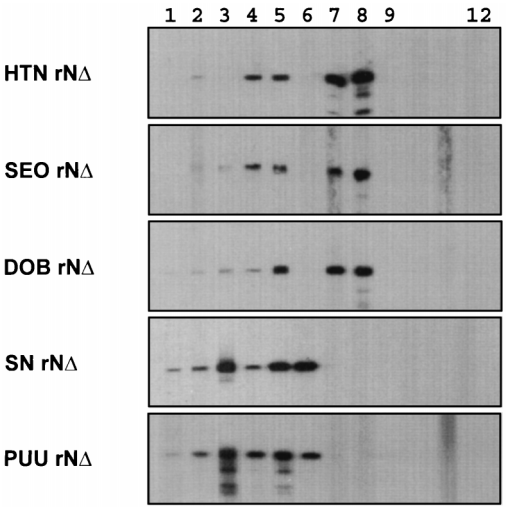

FIG. 3. WB detecting IgG reactivity in sera from hantavirus-infected patients from Sweden (lanes 1 to 4), the United States (lanes 5 and 6), and Korea (lanes 7 and 8) plus sera from four nonimmune blood donors (lanes 9 to 12) to HTN, SEO, DOB, SN, and PUU virus truncated amino-terminal recombinant polyhistidine-containing N (aa's 1 to 117 of N) (rNΔ).

We found that both hantaviruses and hantavirus rNs induced high antibody titers in animals (rabbit and mouse), and the greatest antibody responses were directed to the homologous antigens when we used the rNΔ-based ELISA (Table 2). The antibody activity could generally be grouped into a HTN, SEO, and DOB virus and SN and PUU virus type responses. The rabbit immunized with PUU virus seemed to give a more group-specific response than the animals immunized with PUU virus rN. The differences in the resulting antigen specificity might be a reflection of differences in the tertiary structure of the N amino-terminal region. Epitopes that induced a cross-reactive antibody response may be hidden in the structure of the native viral N but may be exposed in the denatured *E. coli*-expressed rN. Similar differences in the specificities of the antibody responses to native or recombinant PUU virus and Tula virus Ns have previously been reported in both bank voles and mice (28, 31). Furthermore, we also obtained more specific responses when mice were immunized with either the SN virus or the PUU virus rN antigen. The difference in specificity for the PUU virus rN-immunized rabbit versus that for the PUU virus-immunized mouse was 12- to 25-fold when measured as the relative cross-reactivities to the HTN, SEO, and DOB virus rNΔs (Table 2). The anti-SN virus rN mouse serum also showed a high specificity for the homologous antigen. Although these differences in the specificities in the seroresponse of rabbits and mice to rN is intriguing, it is not possible to draw any general conclusions due to the limited number of animals tested.

By using sera from humans infected with hantaviruses, the amino-terminal hantavirus rNΔs were assayed by WB for their usefulness as antigens for the detection of IgG antibodies. We found that the antibody responses to the antigens were specific, but substantial cross-reactivity could be seen within the HTN, SEO, and DOB virus and the SN and PUU virus groups (Fig. 3). The hantavirus-specific response of the sera by WB was compared to that by ELISA by using the same five hantavirus rNΔs and to the results of IFA, which uses virus-infected cells as antigen. As expected, these three methods showed a high degree of correlation (Table 3).

Using the five rNΔ antigens in an ELISA, we were able to detect hantavirus-specific IgM and IgG antibody responses in sera originating from patients with acute or retrospectively diagnosed hantavirus infections from many regions of the

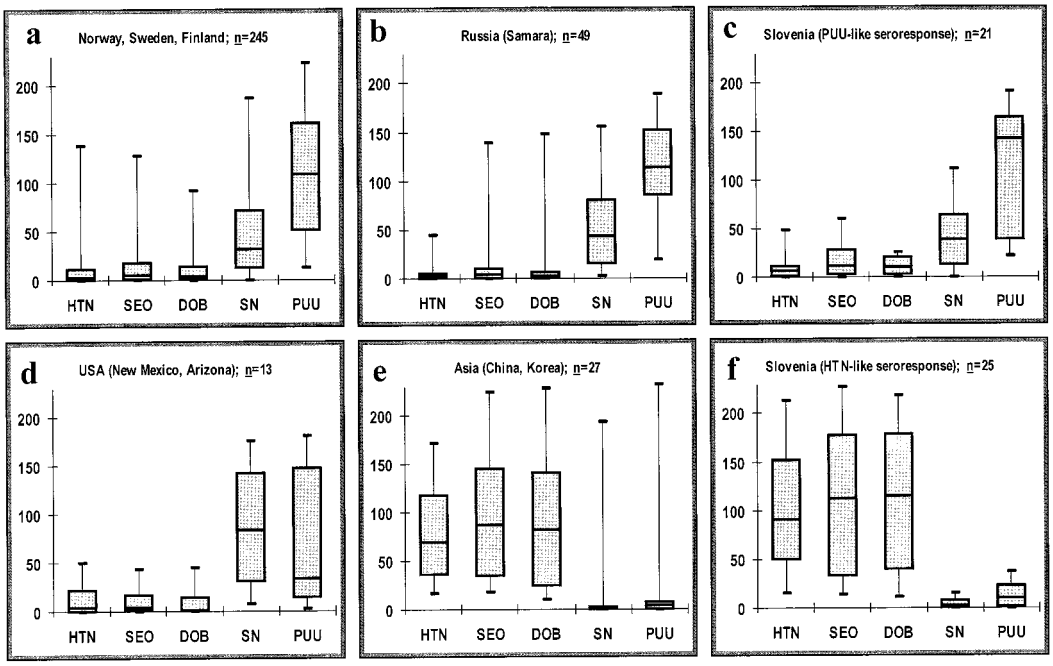

FIG. 4. IgG reactivity in ELISA of sera from patients with an acute or recent hantavirus infection from Scandinavia (Norway, Sweden, and Finland), Russia, Slovenia, the United States, and Asia to truncated amino-terminal recombinant polyhistidine containing N (aa's 1 to 117 of N) specific for HTN, SEO, DOB, SN, and PUU viruses. ELISA values are given as AU. Vertical bars represent the activities of all sera. The boxes include the eight middle percentiles and the median value (horizontal bar).

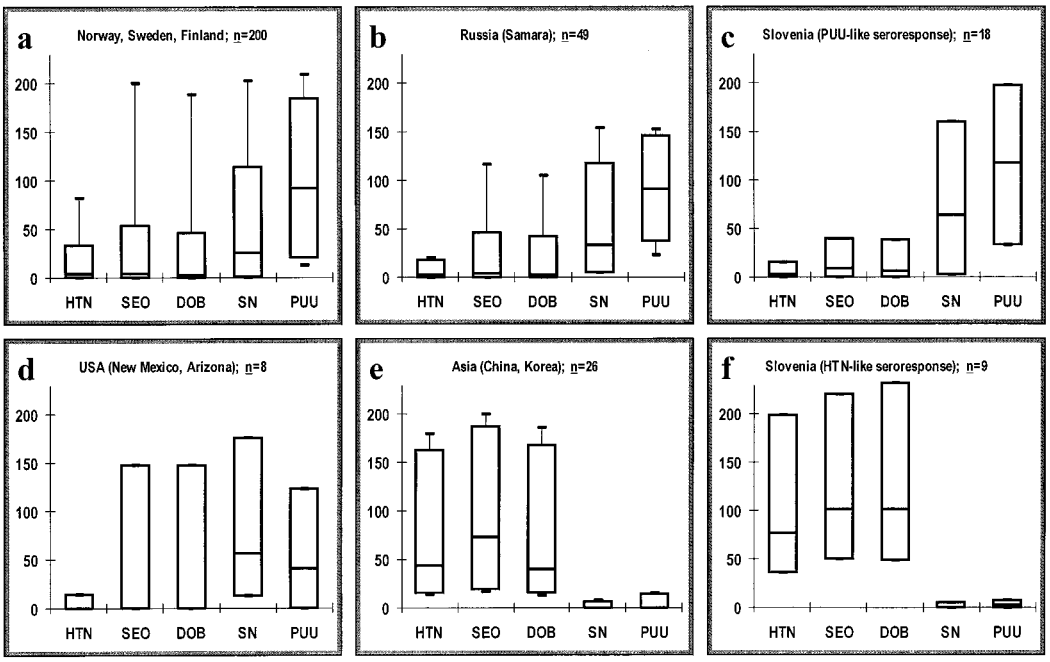

FIG. 5. IgM reactivity by ELISA of sera from patients with an acute or recent hantavirus infection from Scandinavia (Norway, Sweden, and Finland), Russia, Slovenia, the United States, and Asia to truncated amino-terminal recombinant polyhistidine containing N (aa's 1 to 117 of N) specific for HTN, SEO, DOB, SN, and PUU viruses. ELISA values are given as AU. Vertical bars represent the activities of all sera. The boxes include the eight middle percentiles and the median value (horizontal bar).

world in a rapid, specific, and sensitive manner. By linear discriminant analyses we confirmed that most Scandinavian, Russian, and U.S. sera could be classified as reactive to the SN and PUU virus group and most Asian sera could be classified as reactive to the HTN, SEO, and DOB virus group. It is promising that the discrimination can be done by the IgM isotype, which is the basis for serological diagnosis of acute hantavirus disease (Fig. 5) (11, 12, 20, 27, 34). An interesting finding was that both reactivity patterns were encountered in sera from Slovenia (Balkan region). This is a reflection of the circulation of HTN-, SEO-, and DOB-like viruses and of PUU-like viruses in this region (2). Since seroreactivity to either of the two groups of viruses is clearly different, it is possible to determine whether reactivity in a single serum sample is due to a virus from the one group or the other. We found only two exceptions in our material; one Korean serum sample showed high levels of IgG reactivity and one U.S. serum sample had high levels of IgM reactivity to all five hantavirus rNΔs (data not shown). At present, a more accurate virus type-specific diagnosis can be achieved only by a neutralization test (7, 8, 33). A recombinant-expressed SN virus glycoprotein has shown promise in distinguishing SN virus infections from infections by other hantaviruses by immunoblotting (18).

A substantial cross-reactivity between the Ns from different hantavirus serotypes was shown in this study. This could be explained by the presence of at least three distinct hantavirus cross-reactive epitopes in this region of the Ns, which have previously been mapped with MAbs (12, 26, 28, 29). The degree of cross-reactivity to the different strains of virus in different sera can be monitored by the approach described here, in which a diagnostic method that determines seroreactivity to nearly equimolar amounts of antigens derived from five different hantaviruses was developed. It was shown that the seroreactivities to the closely related HTN, SEO, and DOB virus group are almost indistinguishable and that a similar pattern was also observed with the SN and PUU virus group.

We have previously shown that the rN-specific antibody activity of both the IgG and the IgM isotypes of sera from humans with NE is mainly directed to epitopes residing between aa's 7 and 94 (12). Our results were compatible with those which have been shown for SN virus, in which the major antigenic determinants for the humoral immune response to rN was found between aa's 17 and 59 (18). Comparisons of the amino acid sequence from hantavirus N amino-terminal regions (aa's 17 to 59) also show a high degree of homology within the two groups of viruses: 83.7 to 90.7% within the HTN, SEO, and DOB virus group and 74.4% for SN and PUU virus N (Table 1). However, amino acid homologies between the two groups of viruses showed only 44.2 to 46.5% homology. We believe that this relationship within and between the two groups of viruses could be the basis of the serological reactivity pattern that was found in our study.

The one-way cross-reactivity seen for patients infected with PUU virus to the HTN virus serotype (33, 36, 41) was occasionally found in the sera from patients with NE and HPS in our study. Seven percent of the serum samples from patients with NE and HPS revealed an IgG activity of ≥40 AU net optical density to the HTN, SEO, and DOB group of viruses. Sera from patients with high ELISA values to the HTN, SEO, and DOB virus group showed a lower reactivity to PUU and SN virus-infected sera; only 2% had IgG values of ≥40 AU to the latter group. This phenomenon is further illustrated in Table 3 and Fig. 3, in which one selected serum sample from a Swedish patient with HFRS and one serum sample from a U.S. patient with HPS showed strong cross-reactivity to HTN, SEO, and DOB virus antigens by IFA, WB, and ELISA. However, for the majority of the sera tested, the reactivity to one of the two serotype groups was by far the highest.

We have shown that the amino-terminal region (aa's 1 to 117) of rN contains major B-cell epitopes of five different hantaviruses. A serological assay, ELISA, for use in the diagnosis of pathogenic hantavirus was developed by using recombinant proteins from the amino-terminal region of the N protein from five different hantaviruses. The ELISAs based on the use of these antigens was in good agreement with the results obtained by WB and IFA. The assay could detect antibody reactivities in patients with hantavirus infections in various parts of the world. Furthermore, by measuring activities of either the IgG or the IgM isotypes, the assay could distinguish in most patients between infections caused by the HTN, SEO, and DOB virus serotype group and the PUU and SN virus serotype group. The five hantavirus rNΔ-based ELISA is in use for the detection of antibody responses in patients with hantavirus disease and for epidemiological screening of sera from different geographical regions.

ACKNOWLEDGMENTS

This work was supported by Swedish Medical Research Council grant B-93-16X-10382-01A; the Kempe Foundation; the Joint Committee of the Northern Swedish Health Care Region; the Swedish Institute; Förenade Liv Mutual Group Life Insurance Company, Stockholm, Sweden; and U.S. Department of Health and Human Services grant RO1 AI 36336 (to B.H.).

We are indebted to Madeleine Hägglund, Ann-Christin Verlemyr, and Katarina Brus Sjölander for expert technical assistance. We thank C. Schmaljohn of the Department of Molecular Virology, USAMRIID, Ft. Detrick, Frederick, Md., for providing hantavirus cDNA and animal sera, Q.-G. Li and L.-C. Ju for providing sera from patients with HFRS, and T. Yamada and S. Jenison for preparing the maltose-binding protein-SN virus rN fusion protein used to make mouse antibodies.

REFERENCES

1. **Arikawa, J., H. F. Lapenotiere, L. Iacono-Connors, M. L. Wang, and C. S. Schmaljohn.** 1990. Coding properties of the S and the M genome segments of Sapporo rat virus: comparison to other causative agents of hemorrhagic fever with renal syndrome. Virology **176:**114–125.
2. **Avsic-Zupanc, T., M. Likar, S. Novakovic, B. Cizman, A. Kraigher, G. van der Groen, R. Stojanovic, M. Obradovic, A. Gligic, and J. LeDuc.** 1990. Evidence of the presence of two hantaviruses in Slovenia, Yugoslavia. Arch. Virol. Suppl. **1:**87–94.
3. **Avsic-Zupanc, T., A. Toney, K. Anderson, Y. K. Chu, and C. Schmaljohn.** 1995. Genetic and antigenic properties of Dobrava virus: a unique member of the hantavirus genus, family Bunyaviridae. J. Gen. Virol. **76:**2801–2808.
4. **Avsic-Zupanc, T., S. Y. Xiao, R. Stojanovic, A. Gligic, G. van der Groen, and J. W. LeDuc.** 1992. Characterization of Dobrava virus: a Hantavirus from Slovenia, Yugoslavia. J. Med. Virol. **38:**132–137.

4a. **Avsic-Zupanc, T., et al.** Unpublished data.

5. **Brummer-Korvenkontio, M., H. Henttonen, and A. Vaheri.** 1982. Hemorrhagic fever with renal syndrome in Finland: ecology and virology of nephropathia epidemica. Scand. J. Infect. Dis. Suppl. **36:**88–91.
6. **Childs, J. E., T. G. Ksiazek, C. F. Spiropoulou, J. W. Krebs, S. Morzunov, G. O. Maupin, K. L. Gage, P. E. Rollin, J. Sarisky, R. E. Enscore, J. K. Frey, C. J. Peters, and S. T. Nichol.** 1994. Serologic and genetic identification of *Peromyscus maniculatus* as the primary rodent reservoir for a new hantavirus in the southwestern United States. J. Infect. Dis. **169:**1271–1280.
7. **Chu, Y. K., G. Jennings, A. Schmaljohn, F. Elgh, B. Hjelle, H. W. Lee, S. Jenison, T. Ksiazek, C. J. Peters, P. Rollin, and C. Schmaljohn.** 1995. Cross-neutralization of hantaviruses with immune sera from experimentally infected animals and from hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome patients. J. Infect. Dis. **172:**1581–1584.
8. **Chu, Y. K., C. Rossi, J. W. LeDuc, H. W. Lee, C. S. Schmaljohn, and J. M. Dalrymple.** 1994. Serological relationships among viruses in the hantavirus genus, family Bunyaviridae. Virology **198:**196–204.
9. **Cosgriff, T. M.** 1991. Mechanisms of disease in hantavirus infection: pathophysiology of hemorrhagic fever with renal syndrome. Rev. Infect. Dis. **13:**97–107.
10. **Duchin, J. S., F. T. Koster, C. J. Peters, G. L. Simpson, B. Tempest, S. R. Zaki, T. G. Ksiazek, P. E. Rollin, S. Nichol, E. T. Umland, R. L. Moolenaar, S. E. Reef, K. B. Nolte, M. M. Gallaher, J. C. Butler, R. F. Breiman, and the Hantavirus Study Group.** 1994. Hantavirus pulmonary syndrome: a clinical description of 17 patients with a newly recognized disease. N. Engl. J. Med. **330:**949–955.

10a. **Elgh, F.** Unpublished data.

11. **Elgh, F., M. Linderholm, G. Wadell, and P. Juto.** 1996. The clinical usefulness of a Puumala virus recombinant nucleocapsid protein based enzyme-linked immunosorbent assay in the diagnosis of nephropathia epidemica as compared to an immunofluorescence assay. Clin. Diagn. Virol. **6:**17–26.
12. **Elgh, F., Å. Lundkvist, O. A. Alexeyev, G. Wadell, and P. Juto.** 1996. A major antigenic domain of the human humoral response to the Puumala virus nucleocapsid protein is located at the amino-terminus. J. Virol. Methods **59:**161–172.
13. **Elgh, F., G. Wadell, and P. Juto.** 1995. Comparison of the kinetics of Puumala virus specific IgM and IgG antibody responses in nephropathia epidemica as measured by a recombinant antigen-based enzyme-linked immunosorbent assay and an immunofluorescence test. J. Med. Virol. **45:**146–150.
14. **Feldmann, H., A. Sanchez, S. Morzunov, C. F. Spiropoulou, P. E. Rollin, T. G. Ksiazek, C. J. Peters, and S. T. Nichol.** 1993. Utilization of autopsy RNA for the synthesis of the nucleocapsid antigen of a newly recognized virus associated with hantavirus pulmonary syndrome. Virus Res. **30:**351–367.
15. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. Gene **73:**237–244.
16. **Hjelle, B., S. Jenison, N. Torrez-Martinez, T. Yamada, K. Nolte, R. Zumwalt, K. MacInnes, and G. Myers.** 1994. A novel hantavirus associated with an outbreak of fatal respiratory disease in the southwestern United States: evolutionary relationships to known hantaviruses. J. Virol. **68:**592–596.
17. **Hjelle, B., S. A. Jenison, D. E. Goade, W. B. Green, R. M. Feddersen, and A. A. Scott.** 1995. Hantaviruses: clinical, microbiologic and epidemiologic aspects. Crit. Rev. Clin. Lab. Sci. **32:**469–508.
18. **Jenison, S., T. Yamada, C. Morris, B. Anderson, N. Torrez-Martinez, N. Keller, and B. Hjelle.** 1994. Characterization of human antibody responses to Four Corners hantavirus infections among patients with hantavirus pulmonary syndrome. J. Virol. **68:**3000–3006.
19. **Kallio-Kokko, H., O. Vapalahti, K. Hedman, M. Brummer-Korvenkontio, and A. Vaheri.** 1993. Puumala virus antibody and immunoglobulin G avidity assays based on a recombinant nucleocapsid antigen. J. Clin. Microbiol. **31:**677–680.
20. **LeDuc, J. W., T. G. Ksiazek, C. A. Rossi, and J. M. Dalrymple.** 1990. A retrospective analysis of sera collected by the Hemorrhagic Fever Commission during the Korean Conflict. J. Infect. Dis. **162:**1182–1184.
21. **Lee, H. W.** 1982. Hemorrhagic fever with renal syndrome (HFRS). Scand. J. Infect. Dis. Suppl. **36:**82–85.
22. **Lee, H. W., L. J. Baek, and K. M. Johnson.** 1982. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. J. Infect. Dis. **146:**638–644.
23. **Lee, H. W., P. W. Lee, and K. M. Johnson.** 1978. Isolation of the etiologic agent of Korean hemorrhagic fever. J. Infect. Dis. **137:**298–308.
24. **Lee, H. W., and G. van der Groen.** 1989. Hemorrhagic fever with renal syndrome. Prog. Med. Virol. **36:**62–102.
25. **Li, D., A. L. Schmaljohn, K. Anderson, and C. S. Schmaljohn.** 1995. Complete nucleotide sequences of the M and S segments of two hantavirus isolates from California: evidence for reassortment in nature among viruses related to hantavirus pulmonary syndrome. Virology **206:**973–983.
26. **Lundkvist, Å., A. Fatouros, and B. Niklasson.** 1991. Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. J. Gen. Virol. **72:**2097–2103.
27. **Lundkvist, Å., J. Hörling, and B. Niklasson.** 1993. The humoral response to Puumala virus infection (nephropathia epidemica) investigated by viral protein specific immunoassays. Arch. Virol. **130:**121–130.
28. **Lundkvist, Å., H. Kallio-Kokko, K. Brus Sjölander, H. Lankinen, B. Niklasson, A. Vaheri, and O. Vapalahti.** 1996. Characterization of Puumala virus nucleocapsid protein: identification of B-cell epitopes and domains involved in protective immunity. Virology **216:**397–406.
29. **Lundkvist, Å., and B. Niklasson.** 1992. Bank vole monoclonal antibodies against Puumala virus envelope glycoproteins: identification of epitopes involved in neutralization. Arch. Virol. **126:**93–105.
30. **Lundkvist, Å., and B. Niklasson.** 1994. Haemorrhagic fever with renal syndrome and other hantavirus infections. Rev. Med. Virol. **4:**177–184.
31. **Lundkvist, Å., O. Vapalahti, A. Plyusnin, K. Brus Sjölander, B. Niklasson, and A. Vaheri.** 1996. Characterization of Tula virus antigenic determinants defined by monoclonal antibodies raised against baculovirus-expressed nucleocapsid protein. Virus Res. **45:**29–44.

31a. **Lundkvist, Å., et al.** Unpublished data.

32. **Nichol, S. T., C. F. Spiropoulou, S. Morzunov, P. E. Rollin, T. G. Ksiazek, H. Feldmann, A. Sanchez, J. Childs, S. Zaki, and C. J. Peters.** 1993. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science **262:**914–917.
33. **Niklasson, B., M. Jonsson, Å. Lundkvist, J. Hörling, and E. Tkachenko.** 1991. Comparison of European isolates of viruses causing hemorrhagic fever with renal syndrome by a neutralization test. Am. J. Trop. Med. Hyg. **45:**660–665.
34. **Niklasson, B., and T. Kjelsson.** 1988. Detection of nephropathia epidemica (Puumala virus)-specific immunoglobulin M by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **26:**1519–1523.

35. **Niklasson, B., and J. W. LeDuc.** 1987. Epidemiology of nephropathia epidemica in Sweden. J. Infect. Dis. **155:**269–276.
36. **Niklasson, B., E. Tkachenko, A. P. Ivanov, G. van der Groen, D. Wiger, H. K. Andersen, J. LeDuc, T. Kjelsson, and K. Nyström.** 1990. Haemorrhagic fever with renal syndrome: evaluation of ELISA for detection of Puumala-virus-specific IgG and IgM. Res. Virol. **141:**637–648.
37. **Schmaljohn, C. S., J. Arikawa, S. E. Hasty, L. Rasmussen, H. W. Lee, P. W. Lee, and J. M. Dalrymple.** 1988. Conservation of antigenic properties and sequences encoding the envelope proteins of prototype Hantaan virus and two virus isolates from Korean haemorrhagic fever patients. J. Gen. Virol. **69:**1949–1955.
38. **Schmaljohn, C. S., G. B. Jennings, J. Hay, and J. M. Dalrymple.** 1986. Coding strategy of the S genome segment of Hantaan virus. Virology **155:** 633–643.
39. **Settergren, B.** 1991. Nephropathia epidemica (hemorrhagic fever with renal syndrome) in Scandinavia. Rev. Infect. Dis. **13:**736–744.
40. **Sheshberadaran, H., B. Niklasson, and E. A. Tkachenko.** 1988. Antigenic relationship between hantaviruses analysed by immunoprecipitation. J. Gen. Virol. **69:**2645–2651.
41. **Svedmyr, A., P. W. Lee, D. C. Gajdusek, C. J. Gibbs, Jr., and K. Nyström.** 1980. Antigenic differentiation of the viruses causing Korean haemorrhagic fever and epidemic (endemic) nephropathy of Scandinavia. Lancet **2:**315–316.
42. **Vapalahti, O., H. Kallio-Kokko, E. M. Salonen, M. Brummer-Korvenkontio, and A. Vaheri.** 1992. Cloning and sequencing of Puumala virus Sotkamo strain S and M RNA segments: evidence for strain variation in hantaviruses and expression of the nucleocapsid protein. J. Gen. Virol. **73:**829–838.
43. **Vapalahti, O., Å. Lundkvist, H. Kallio-Kokko, K. Paukku, I. Julkunen, H. Lankinen, and A. Vaheri.** 1996. Antigenic properties and diagnostic potential of Puumala virus nucleocapsid protein expressed in insect cells. J. Clin. Microbiol. **34:**119–125.
44. **Wang, M., C. Rossi, and C. S. Schmaljohn.** 1993. Expression of non-conserved regions of the S genome segments of three hantaviruses: evaluation of the expressed polypeptides for diagnosis of haemorrhagic fever with renal syndrome. J. Gen. Virol. **74:**1115–1124.
45. **Zöller, L., S. Yang, P. Gött, E. K. Bautz, and G. Darai.** 1993. Use of recombinant nucleocapsid proteins of the Hantaan and nephropathia epidemica serotypes of hantaviruses as immunodiagnostic antigens. J. Med. Virol. **39:**200–207.